STUDY BY COMPLEMENT FIXATION OF HYPERIMMUNE SERA TO MYCOPLASMAS PRODUCED IN RABBITS MADE TOLERANT TO MEDIA COMPONENTS

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Complement fixation tests have been used in identifying strains of Mycoplasma spp. as well as in studying antigenic relationships between heterologous strains. However, there is a major hindrance in the interpretation of most serological tests involving Mycoplasma spp., that being medium antibodies which are formed along with antibodies to mycoplasma antigens in the production of hyperimmune sera. This results in spurious cross reactions through the medium antibodies reacting with medium components on the test antigens. It has been shown that the standard procedure for washing mycoplasma antigens, three washings in phosphate buffered saline, is not sufficient to remove media from certain strains.

Most strains of Mycoplasma spp. require serum supplement in the medium in which they are grown, and most medium antibodies appear to be directed against serum antigens. Many workers have tried to avoid medium antibodies by using serum supplement from species or individuals being used for the production of hyperimmune sera. Generally, however, not all strains of Mycoplasma spp. under study will grow optimally in medium with one serum supplement, e.g., one strain may grow well in medium supplemented with swine serum but not rabbit serum, and vice versa for another strain. Also, some researchers have detected isoantibodies and antibodies against other components of the media besides the serum fraction.

The research reported herein was an attempt to prevent the problem of medium antibodies by producing tolerance in rabbits to the media used

to propagate the Mycoplasma spp., thus resulting in production of antibodies to the mycoplasma immunogens alone.

REVIEW OF THE LITERATURE

Non-Specific Serological Reactions

Edward and Kanarek (8) in 1960 first postulated that non-specific serological reactions may occur if Mycoplasma spp. which are to be used for inoculation of rabbits are grown in a medium supplemented with heterologous sera. That rabbits would produce antibodies to the foreign serum as well as to the mycoplasma antigens was theoretically possible, and, although, no evidence was cited, it was suggested that washing of the antigen might not rid it of the serum. To eliminate the problem, the authors used horse serum supplemented media for the growth of test antigens and rabbit serum supplemented media for the production of antigen to be inoculated into rabbits.

In 1966 Smith, Dunlap, and Stroud (22) reported that the culture medium did, indeed, affect the apparent serological relationships of mycoplasma strains. The results of the growth inhibition test using two strains of Mycoplasma gallisepticum were dependent on the type of media in which the two strains were grown. Two different kinds of media were used. One was supplemented with bovine serum fraction and the other, termed enriched medium, was supplemented with horse serum, yeast hydrolysate, and glucose. The strains appeared to be serologically identical when grown in the non-enriched medium. However, one strain, when grown in the enriched medium, was inhibited by hyperimmune sera prepared from the homologous strain grown in the enriched but not the non-enriched

medium. It was not inhibited by hyperimmune serum prepared from the heterologous strain grown in either broth. The results were the same regardless of whether the antigens used for the production of hyperimmune sera were unwashed or washed three times in phosphate buffered saline. An additional finding was that hyperimmune serum to the enriched broth alone effectively inhibited both strains when they were grown in the enriched medium. Hyperimmune sera to the non-enriched broth was not inhibitory in any instance.

Further investigation of the ability of Mycoplasma gallisepticum to adsorb medium components was reported in 1968 by Jordan and Kulasegaram (14). Two medium components, swine serum and peptone, were found to be capable of eliciting antibody responses in chickens and rabbits when these animals were inoculated with Mycoplasma gallisepticum grown in media containing these components, and the antibodies were demonstrable by gel diffusion even after the inoculating antigen was washed twelve times in phosphate buffered saline. They proposed using tissue infusion media homologous to the species of animal to be inoculated for the production of hyperimmune sera; the possibility of antibodies to alloantigens being formed in this type of procedure was not ruled out.

The adsorption of medium proteins to Mycoplasma gallisepticum at an acid pH was demonstrated by Bradbury and Jordan (3) in 1971. No adsorption was detectable by gel diffusion tests with organisms grown in buffered medium in which the pH remained neutral. Antibodies to the serum supplement, but no other medium component, were observed. By electrophoretically studying the sera from rabbits injected with Mycoplasma gallisepticum grown in medium supplemented with swine serum, three precipitins were found consistently: one each to swine serum IgG, IgM, and

a protein of alpha-globulin mobility. It was concluded that Mycoplasma gallisepticum preferentially adsorbed these swine serum components since there was an absence of antibody to albumin which is a good immunogen and constitutes approximately one-third of the total protein of swine serum. Similar results were obtained using chicken sera instead of swine sera.

It has been demonstrated that Mycoplasma gallisepticum grown in broth with an acid pH for longer than eight hours will have a decrease in sensitivity to the hemagglutination and serum plate agglutination tests (3). This implies that the adsorption of media components by the organism, besides causing reactions with antisera specific for these media components, could alter the specificity of immunogens associated with the organism by changing or masking the antigenic determinants on the immugen resulting in non-specific reactions or the loss of serologic activity.

There are numerous other reports in the literature of cross reactions involving Mycoplasma gallisepticum in agglutination tests but many of them are difficult to explain on the basis of adsorbed media components. The problem becomes quite complex due to contradictory results involving these cross reactions. Cross reactions to Mycoplasma gallisepticum antigen in the serum plate agglutination test have occurred with sera from chickens experimentally infected with Mycoplasma synoviae (17, 18), Erysipelothrix bacterin (2, 18), avian mycoplasma serotype P (18), Streptococcus faecalis (24), and Staphylococcus aureus (24). Cross reactions to Mycoplasma gallisepticum in agglutination tests have also occurred with sera from chickens that had recently been inoculated with commercial inactivated vaccines of avian encephalomyelitis, Newcastle disease, and infectious bronchitis viruses (20, 27). Freezing and thawing (5, 23), and the contamination (19) of negative sera have also been

shown to cause false positive reactions. It should be noted that all the cross reactions have occurred in the serum plate agglutination test; the same sera remained specific in the hemagglutination-inhibition test (18, 20).

Winsdor and Thornton (27) propagated Mycoplasma gallisepticum antigen in globulin-free media in an attempt to avoid the non-specific reactions which occurred apparently as a result of the combination of antigen-adsorbed globulins and globulin antibodies produced following various infections or immunizations. Two later published reports have not substantiated the value of using globulin-free media. Bradbury and Jordan (5) were not able to correlate the presence of globulin antibodies with non-specific activity or eliminate non-specific reactions by the use of globulin-free media. Kleven (15) also reported that globulin-free antigens were still capable of non-specific reactions. It thus appears that other factors, besides the adsorption of gamma globulins from the media, may play a role in non-specific serum plate agglutination reactions.

Production of Tolerance

Tolerance is the inability of the immune system to respond to a specific immunogen. This phenomenon was demonstrated in rabbits in 1954 by Hanan and Oyama (12) in an experiment designed to study the effects of prenatal and post-natal immunization of rabbits with bovine serum albumin (BSA) on the antibody and gamma globulin content of the serum. It was discovered that those rabbits which received BSA right after birth failed to make precipitating antibodies to BSA when challenged later in life, although their serum gamma globulin levels were equivalent to that of the

control rabbits.

Cinader and Dubert (7) produced tolerance to albumin of human origin by giving neonatal rabbits multiple injections of the antigen; this state of tolerance lasted for at least eight months after birth. Antibody responses to unrelated antigen, tobacco mosaic virus, were not affected, but varying responses were obtained when the rabbits were challenged with diazo-human albumin (diazo compound of benzene- p-sulphonic acid coupled with purified albumin of human origin). Two of six tolerant rabbits did produce antibody to diazo-human albumin, although the antibodies were shown to be directed against the benzene- p-sulphonic acid group, not the albumin molecule.

In 1958 Smith and Bridges (21) defined more closely the factors necessary for producing the tolerant state in neonatal rabbits. It was shown that a single, intraperitoneal injection on the day of birth of between 10 mg and 100 mg BSA or ovalbumin induced a state of unresponsiveness lasting at least 90 to 120 days in ninety per cent of the rabbits. After 17 days of age, the administration of 100 mg BSA failed to produce tolerance in any of the rabbits tested. It was also found that once the unresponsive state was established, it could be perpetuated by subsequent injections of small doses given in adult life, generally at three month intervals.

The method of eliminating cross reactions due to adsorbed media components by producing tolerance to these components was first demonstrated by Frey, Thomas, and Hale (9) in 1973. The injection of 0.25 ml of broth medium plus 0.25 ml of serum supplement into rabbits intraperitoneally within twelve hours after birth resulted in almost all the rabbits

producing no medium antibodies when challenged later in life with washed mycoplasma organisms.

MATERIALS AND METHODS

Mycoplasma Strains

The four mycoplasmas used in this study were reference strains supplied by Dr. M. L. Frey. Three strains were originally isolated from cattle: B-600 (M. bovirhinis), B-761 (M. alkalescens), and B-762 (Mycoplasma sp.). The fourth strain, PG-31 (M. gallisepticum) is an avian strain and was used in this study because of previous work demonstrating that it could adsorb media components.

All strains were originally cloned by filtration of the culture in broth medium through a 300 nm or a 450 nm membrane filter, culturing the filtrate on solid medium, and picking an isolated colony to repropagate in broth medium. This procedure was done three times to obtain the final cloned reference organisms.

Mycoplasma Media

Each strain of mycoplasma was propagated in five different kinds of media: AOHO, AORA, AOSW, VFHO, and PHO.

The basic AO medium was made by dissolving 4.0 gm peptone CS,[a] 2.0 gm peptone B,[a] 2.0 gm peptone M,[a] 2.0 gm yeast autolysate,[a] 2.0 gm yeast extract,[a] 5.0 gm NaCl, 0.4 gm KCl, and 0.2 gm $MgSO_4 \cdot 7H_2O$ in one liter of distilled, deionized water. The pH was adjusted to 7.5 with

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[a]Pfizer, Inc., New York, New York 10017.

1N NaOH, and the broth was sterilized by autoclaving at 15 pounds pressure for fifteen minutes. This medium is part of a more complex medium, M-96 basal medium, used for the isolation and growth of more fastidious mycoplasmas (9). The three types of AO media were made by adding 16% horse serum,[b] 16% rabbit serum,[c] and 16% swine serum, respectively, to aliquots of the basic AO broth. Swine sera was obtained from swine at slaughter and acid-treated by the method of Boulanger (1).

The PHO medium was made by dissolving 21.0 gm PPLO[d] broth medium and 1% yeast autolysate[e] in one liter of distilled, deionized water and adjusting the pH to 7.5 with 1N NaOH. The broth was then supplemented with 16% horse serum.[b]

VFHO medium is viande foie broth with 16% horse serum.[b] The viande foie broth was prepared by the method described by Turner, et al. (25) and is a digest medium prepared with beef muscle, beef liver, and swine gastric mucosa.

No selective bacterial inhibitors were added to the media; sterility of the media was ascertained by inoculating PHO agar plates with a loopful of the broth suspension and observing the plates for growth grossly and microscopically after 24, 48, and 72 hours' incubation at 37 °C.

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[b] International Scientific Industries, Inc., Cary, Illinois 60013.

[c] Pel-Freez Biologicals, Inc., Rogers, Arkansas 72756.

[d] Difco Laboratories, Detroit, Michigan 48232.

[e] Pfizer, Inc., New York, New York 10017.

Antigens

Each of the four Mycoplasma spp. were inoculated into broth tubes of the different media and passaged in these media at 24 hour intervals for several days at which time good growth was obtained when a small loopful of broth was transferred to PHO agar. Then 500 ml volumes of each media were inoculated with 2 ml of broth culture from the appropriate tubes. These were incubated at 37 °C. until visible growth could be observed by the turbidity of the broth (between 48 and 72 hours). The large volumes of broth cultures were tested for growth and contamination by plating a loopful of the culture on PHO agar and observing for good growth of mycoplasma colonies and the absence of bacterial and fungal growth. Three hundred ml of each broth culture was used for hyperimmunization antigen production and two hundred ml for production of test antigen.

The hyperimmunization antigen was prepared by centrifugation of the 300 ml broth aliquot at 5000 x g for thirty minutes. The resultant pellet was resuspended in 150 ml of the respective broth giving a 2x concentration of the antigen in the appropriate broth. These antigens were used for the injection of rabbits and were stored at 4 °C. and checked weekly for contamination by inoculating a loopful of the suspension on PHO agar plates.

The mycoplasma cultures to be used as test antigens were centrifuged at 5000 x g for thirty minutes. The resultant pellet was resuspended in sterile phosphate buffered saline (0.185% NaCl in 0.01 M phosphate buffer at pH 7.4) and recentrifuged. This washing was done three times, and the final pellet was resuspended in 7 to 15 ml of phosphate buffered saline. Sterile glycerol was then added equal to 25% of the volume of antigen; the final antigen preparation was stored at -10 °C.

Rabbits

Preparations were made in advance with two local rabbit breeding operations to inject litters of rabbits at birth and at 4 weeks of age. In addition, two litters used in this study were kindled at the O.S.U. College of Veterinary Medicine.

Each litter was injected with one type of media. All litters were injected within 24 hours after birth, and the majority of litters within 12 hours. Two rabbits from each litter were not inoculated at birth nor at 4 weeks of age; these rabbits served as controls. Each rabbit injected at birth was given 0.3 ml of the appropriate broth intraperitoneally. The injections were repeated when the rabbits were 4 weeks of age using 0.5 ml of the appropriate broth intraperitoneally. Control rabbits of each litter were identified by color, markings, or ear notch.

At eight weeks of age, the rabbits were brought to the College laboratory animal facilities, and each was given an identification tattoo number in the right ear. A serum sample was obtained from each rabbitt at this time by cardiac bleeding. All rabbits were given 1.0 ml of the appropriate broth or mycoplasma-broth mixture three times per week intraperitoneally for a total of six weeks. The rabbits were bled intracardially one week after the last injection. The serum was stored at -10 °C. without preservatives.

Complement Fixation Test

Each serum sample obtained at the final bleeding was tested against each mycoplasma antigen grown in the various broths by the microtiter complement fixation test. The procedure used followed the Laboratory Branch Complement Fixation Method (6).

Sheep red blood cells were collected from adult, healthy sheep at the College. Sixty ml of sheep blood were taken aseptically from the jugular vein and collected in 100 ml of sterile modified Alsever's solution. The blood solution was then agitated gently for ten minutes and stored at 4 °C. Sheep blood was used only after a minimum of five days of storage at 4 °C. and up to five weeks after collection.

Commercial hemolysin[f] and complement[g] were used throughout the test. Standardization of sheep erythrocytes was carried out by the centrifugation method (6).

Each antigen was titrated using the microtiter technique against its specific antiserum. The optimal antigen dilution was the dilution which gave the greatest amount of fixation with its antiserum and displayed no anticomplementary activity. This antigen dilution was used in the complement fixation test proper.

Each antiserum was tested for anticomplementary activity, and those antisera demonstrating such activity were treated by adding 0.15 ml of undiluted guinea pig complement to 0.6 ml of antiserum (16). This mixture was held overnight at 4 °C. The next day the mixture was diluted 1:16 with veronal buffered diluent and heated at 56 °C. for thirty minutes before being used in the test as were all serum samples tested.

The final titer determined for each sample was the reciprocal of the highest dilution of antiserum which showed 30% or less hemolysis. Doubling dilutions of antisera were used in the test beginning at 1/16 and continuing through 1/256. Several serum samples were shown to be

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[f]Microbiological Associates, Inc., Bethesda, Maryland 20014.

[g]Flow Laboratories, Rockville, Maryland 20852.

anticomplementary at the 1/16 dilution even after being treated for such activity; however, no sera were anticomplementary at the 1/32 dilution. Thus, the test results are in the range of 32 to 256 since activity at dilutions less than 1/32 was uninterpretable in some instances.

Gel Diffusion Precipitin Test

Each antiserum was tested against each antigen by the gel diffusion precipitin test. The agar was prepared by adding 0.9% Special Agar-Noble[h] and 8.5% NaCl to distilled water (13). A few crystals of trypan blue were added to give the medium a pale blue color for easier reading of the precipitation lines. The melted agar was dispensed into 5 cm diameter petri dishes in 7 ml aliquots. Wells, 4 mm in diameter with centers 12 mm apart and numbering from 5 to 7, were cut in each dish of agar. The wells were filled with the appropriate antisera or antigens; the plates were incubated at 37 °C. and read daily for one week.

[h]Difco Laboratories, Detroit, Michigan 48232.

RESULTS

The complement fixation titers of all the antisera which were tested against all antigens are shown in Tables 1-5. Table 6 lists the tolerant and non-tolerant antisera produced to the four Mycoplasma spp. and their resultant C-F titers to the various mycoplasma antigens. It can be seen from these tables that those rabbits which appeared to develop tolerance to the media components still had antibodies capable of cross reacting with heterologous strains. The cross reactions observed were not consistent. All strains appeared to cross react with the heterologous strains to some degree. The cross reaction of strains B-761 and B-762 was the most pronounced, the two strains appearing identical in these tests. The least amount of cross reactivity was demonstrated between strains B-600 and PG-31; PG-31 antisera had a higher percentage of cross reactivity with B-600 antigens than the B-600 antisera with the PG-31 antigens.

The comparison of sera from rabbits which were injected neonatally and from rabbits which were not injected until eight weeks of age, both only having been injected with broth media throughout the experiment, is shown in Table 7. Only 4 of 7 rabbits which were exposed neonatally actually demonstrated tolerance to the media with which they were injected. Two rabbits which had undergone identical injections with AORA broth medium responded quite differently to the medium antigens; neither rabbit showed complete tolerance but antiserum from one rabbit reacted to 19 of the 25 antigens, while antiserum from the other rabbit reacted to only 3

TABLE 1--C-F Titers of AOHO Antisera and Antigens

ANTIGENS	ANTISERA						
	B-600(2)*	B-761	B-762	PG-31(2)	AOHO	AOHO-C**	PG-31C
AOHO	— — —	— — —	— — —	— — —	— — —	[128]	[32]
B-600	256; -	— — —	— — —	— — —	— — —	32	— — —
B-761	— — —	— — —	— — —	— — —	— — —	32	— — —
B-762	— — —	— — —	— — —	— — —	— — —	— — —	— — —
PG-31	— — —	— — —	— — —	— — —	— — —	128	32
AORA	— — —	— — —	— — —	— — —	— — —	— — —	— — —
B-600	[256+]; 32	64	32	— — —	— — —	— — —	— — —
B-761	— — —	256+	256+	— — —	32	— — —	32
B-762	— — —	256+	256+	— — —	128	— — —	128
PG-31	— — —	— — —	— — —	— — —	64	— — —	— — —
AOSW	— — —	— — —	— — —	— — —	— — —	— — —	— — —
B-600	— — —	— — —	— — —	128; -	— — —	— — —	— — —
B-761	64; 32	128	256+	64; 32	64	— — —	32
B-762	— — —	32	32	64; -	— — —	— — —	— — —
PG-31	— — —	— — —	256+	64; 256+	— — —	— — —	— — —
VFHO	— — —	— — —	— — —	— — —	— — —	[64]	— — —
B-600	— — —	— — —	— — —	— — —	— — —	128	— — —
B-761	— — —	— — —	— — —	— — —	— — —	32	— — —
B-762	— — —	— — —	— — —	— — —	— — —	32	64
PG-31	— — —	— — —	— — —	— — —	— — —	32	32
PHO	[—]	— — —	[32]	— — —	— — —	[128]	[32]
B-600	— — —	— — —	— — —	— — —	— — —	— — —	— — —
B-761	— — —	256	64	— — —	32	64	32
B-762	32	256+	256+	— — —	32	[256+]	64
PG-31	— — —	— — —	— — —	— — —	— — —	64	32

*DENOTES SERUM SAMPLES TAKEN FROM DIFFERENT RABBITS.

**DENOTES ANTISERA FROM CONTROL RABBITS (NOT INJECTED UNTIL 8 WEEKS OF AGE).

[ ] DENOTES ANTISERA WHICH FORMED LINES OF PRECIPITATION WITH ANTIGEN IN THE GEL DIFFUSION PRECIPITIN TEST.

TABLE 2--C-F Titers of AORA Antisera and Antigens

ANTIGENS	ANTISERA						
	B-600(3)*	B-761(2)	B-762(2)	PG-31(3)	AORA(2)	AORA-C**	B-600-C(2)
AOHO	— — —	—; 32	— — —	— — —	— — —	— — —	—; 32
B-600	[32;128] 64	—;256+	—; 32	— — —	—; 32	— — —	[—]; 32
B-761	— — —	—;256+	—; 64	— — —	—; 32	— — —	—; 32
B-762	— — —	—;256+	32; 64	— — —	—; 32	— — —	—; 32
PG-31	— — —	—; 32	— — —	—;32; 32	—; 32	— — —	—; 32
AORA	— — —	— — —	— — —	— — —	— — —	— — —	— — —
B-600	256+;128; 64	32;256+	64; —	— — —	— — —	— — —	128;256+
B-761	— — —	256+128	256+; 64	— — —	— — —	32	— — —
B-762	256+;—;—	256+	256+; 64	64;—;—	64; 32	32	128; 32
PG-31	— — —	—; 32	— — —	—[128] 64	—; 32	— — —	—; 32
AOSW	— — —	—; 32	— — —	— — —	—; 32	— — —	—; 32
B-600	—;—; 32	—; 64	—; 32	—;32; 64	—; 32	— — —	—; 64
B-761	64;32;32	256+	256+; 64	32	64	128	64
B-762	—;—; 32	—; 32	—; 32	32;—; 32	—; 32	32	—; 64
PG-31	— — —	—; 32	—; 32	[32;64] 32	—; 32	32	—; 64
VFHO	— — —	— — —	— — —	— — —	— — —	— — —	— — —
B-600	—;32;—	—; 32	—; 32	— — —	—; 32	— — —	—; 32
B-761	— — —	—; 32	—; 32	— — —	—; 32	— — —	—; 32
B-762	— — —	—; 32	— — —	— — —	—; 32	— — —	—; 32
PG-31	— — —	—; 32	— — —	— — —	— — —	— — —	—; 32
PHO	— — —	—; 32	— — —	— — —	—; 32	— — —	—; 32
B-600	— — —	—; 32	— — —	— — —	—; 32	— — —	—; 32
B-761	— — —	128;256+	128; 64	—;—; 32	—; 32	32	—; 32
B-762	32;—;—	256+	256+;128	64;—;—	32	64	— — —
PG-31	—;—;32	—; 32	—; 32	32	—; 32	— — —	—; 32

*DENOTES SERUM SAMPLES TAKEN FROM DIFFERENT RABBITS.
**DENOTES ANTISERA FROM CONTROL RABBITS (NOT INJECTED UNTIL 8 WEEKS OF AGE).
[ ] DENOTES ANTISERA WHICH FORMED LINES OF PRECIPITATION WITH ANTIGEN IN THE GEL DIFFUSION PRECIPITIN TEST.

TABLE 3--C-F Titers of AOSW Antisera and Antigens

ANTIGENS	ANTISERA						
	B-600(2)*	B-761	B-762(2)	PG-31(2)	AOSW(2)	AOSW-C **	PG-31C
AOHO	— — —	— — —	— — —	— — —	— — —	32	32
B-600	—, 32	— — —	32	— — —	32,—	32	32
B-761	— — —	— — —	—,32	— — —	— — —	32	32
B-762	— — —	64	—,32	— — —	— — —	32	64
PG-31	— — —	— — —	— — —	— — —	— — —	32	32
AORA	— — —	— — —	— — —	— — —	— — —	— — —	— — —
B-600	64	— — —	64, 32	[256+]	— — —	32	32
B-761	— — —	32	— — —	32, —	— — —	— — —	— — —
B-762	32,—	64	32	128, —	— — —	32	32
PG-31	—, 32	32	— — —	32, 64	— — —	32	128
AOSW	32,—	— — —	32, —	64, —	32	[128]	[64]
B-600	— — —	— — —	—, 32	— — —	32,—	64	64
B-761	64; 32	64	32, 64	128; 32	32,—	64	64
B-762	32,—	32	64, 32	32	64,—	64	64
PG-31	— — —	— — —	32, —	64;[—]	32	[128]	64
VFHO	— — —	— — —	— — —	— — —	— — —	— — —	32
B-600	—, 32	32	—,32	—,32	32	32	64
B-761	— — —	— — —	— — —	— — —	— — —	— — —	32
B-762	— — —	— — —	— — —	— — —	32,—	32	32
PG-31	— — —	— — —	— — —	— — —	— — —	32	32
PHO	— — —	— — —	— — —	— — —	— — —	— — —	32
B-600	— — —	— — —	—,32	— — —	— — —	32	32
B-761	32	64	—,32	32, —	— — —	32	32
B-762	32, —	128	32	32,[—]	32, —	32	32
PG-31	— — —	— — —	—,32	— — —	32, —	— — —	32

*DENOTES SERUM SAMPLES TAKEN FROM DIFFERENT RABBITS.

**DENOTES ANTISERA FROM CONTROL RABBITS (NOT INJECTED UNTIL 8 WEEKS OF AGE).

[ ] DENOTES ANTISERA WHICH FORMED LINES OF PRECIPITATION WITH ANTIGEN IN THE GEL DIFFUSION PRECIPITIN TEST.

TABLE 4--C-F Titers of VFHO Antisera and Antigens

ANTIGENS	ANTISERA					
	B-600	B-761	PG-31	VFHO	VFHO-C *	PG-31C
AOHO	— — —	[128]	[256+]	— — —	[256+]	[256+]
B-600	64	[256+]	[256+]	32	[64]	[256+]
B-761	— — —	128	128	— — —	64	256+
B-762	— — —	64	64	— — —	[64]	[128]
PG-31	— — —	64	128	— — —	[128]	[256+]
AORA	— — —	— — —	32	— — —	32	— — —
B-600	256+	256+	64	— — —	64	— — —
B-761	32	128	64	32	64	— — —
B-762	256	256+	256	64	256+	64
PG-31	— — —	— — —	[32]	— — —	32	[—]
AOSW	— — —	[32]	[64]	— — —	32	[32]
B-600	— — —	— — —	64	— — —	128	32
B-761	64	256+	256+	64	256+	128
B-762	32	128	256+	32	256+	128
PG-31	— — —	32	64	— — —	32	[32]
VFHO	— — —	[64]	[256]	— — —	[128]	[256+]
B-600	32	256+	[256+]	— — —	128	256+
B-761	— — —	64	128	— — —	— — —	256+
B-762	32	64	128	— — —	128	256+
PG-31	32	64	256	— — —	128	256+
PHO	[64]	[256+]	[256+]	[64]	[256+]	[256+]
B-600	— — —	32	64	— — —	64	64
B-761	64	256+	256+	32	256+	256+
B-762	32	[256+]	[256+]	32	256+	[256+]
PG-31	— — —	256+	256+	64	256+	256+

*DENOTES SERUM SAMPLES TAKEN FROM DIFFERENT RABBITS.

[ ] DENOTES ANTISERA WHICH FORMED LINES OF PRECIPITATION WITH ANTIGEN IN THE GEL DIFFUSION PRECIPITIN TEST.

TABLE 5--C-F Titers of PHO Antisera and Antigens

ANTIGENS	ANTISERA						
	B-600(2)*	B-761(2)	B-762(2)	PG-31(2)	PHO	PHO-C**	PG-31C
AOHO	[—], [32]	— — —	[—], —	— — —	— — —	— — —	[256]
B-600	256+, 128	—, 64	64, 32	64, 32	32	64	[128]
B-761	—, 32	32, 256+	256+, 128	32, —	— — —	64	64
B-762	—, 32	64, 256+	256	32, —	— — —	32	64
PG-31	—, 32	— — —	— — —	—, 32	— — —	32	64
AORA	— — —	— — —	— — —	— — —	— — —	— — —	— — —
B-600	[256+], 128	[256+], 64	32	128	— — —	— — —	— — —
B-761	128, —	256+, 256	256, 128	256+, —	— — —	— — —	— — —
B-762	128, —	256+	256, 128	256+, —	— — —	32	— — —
PG-31	— — —	— — —	— — —	[—], [—]	— — —	— — —	— — —
AOSW	— — —	— — —	— — —	— — —	— — —	— — —	— — —
B-600	32	— — —	— — —	— — —	— — —	— — —	32
B-761	—, 32	256+, 256	32, 64	32	32	64	32
B-762	32, 64	128, 32	32, —	32, —	— — —	— — —	32
PG-31	32, —	32, —	— — —	32, [32]	— — —	— — —	32
VFHO	— — —	— — —	— — —	— — —	— — —	— — —	[64]
B-600	32	— — —	— — —	32, —	— — —	64	128
B-761	32, —	— — —	— — —	32, —	— — —	32	64
B-762	64, —	— — —	— — —	32, —	— — —	32	64
PG-31	32, —	32, —	— — —	64, —	— — —	32	64
PHO	—, [32]	32	—, [—]	32, [32]	32	[256+]	[128]
B-600	32, —	— — —	— — —	32, —	— — —	— — —	32
B-761	64, —	256, 256+	256+, 128	64, —	32	32	64
B-762	64, 32	256+	256+	256, —	— — —	64	[128]
PG-31	32	—, 32	— — —	32, 64	— — —	128	256

*DENOTES SERUM SAMPLES TAKEN FROM DIFFERENT RABBITS.
**DENOTES ANTISERA FROM CONTROL RABBITS (NOT INJECTED UNTIL 8 WEEKS OF AGE).
[ ] DENOTES ANTISERA WHICH FORMED LINES OF PRECIPITATION WITH ANTIGEN IN THE GEL DIFFUSION PRECIPITIN TEST.

TABLE 6--C-F Titers of Tolerant and Non-Tolerant Antisera to Homologous and Heterologous Mycoplasma Antigens

ANTIGENS	ANTISERA											
	B-600			B-761			B-762			PG-31		
	T*	C†	NT**	T	C	NT	T	C	NT	T	C	NT
B-600	22/40‡	27/50	5/10	8/20	15/35	7/15	14/30	16/35	2/5	4/30	15/50	11/20
B-761	13/40	17/50	4/10	14/20	27/35	13/15	22/30	23/35	1/5	7/30	21/50	14/20
B-762	13/40	19/50	6/10	14/20	27/35	13/15	21/30	24/35	3/5	6/30	19/50	13/20
PG-31	6/40	8/50	2/10	6/20	13/35	7/15	4/30	5/35	1/5	13/30	26/50	13/20

* T DENOTES ANTISERA FROM RABBITS TOLERANT TO MEDIA COMPONENTS.

** NT DENOTES ANTISERA FROM RABBITS NOT TOLERANT TO MEDIA COMPONENTS.

† C DENOTES COMBINED TOTAL OF TOLERANT AND NON-TOLERANT ANTISERA.

‡ NUMBER OF POSITIVE ANTISERA OVER NUMBER OF ANTISERA TESTED.

TABLE 7--Comparison of Sera From Rabbits Injected Neonatally and Rabbits Injected at 8 Weeks of Age With Broth Media

ANTIGENS †	ANTISERA									
	AOHO-T*	AOHO-C**	AORA-T	AORA-C	AOSW-T	AOSW-C	VFHO-T	VFHO-C	PHO-T	PHO-C
AOHO	0/5‡	4/5	0/5 4/5	0/5	1/5 0/5	5/5	1/5	5/5	1/5	4/5
AORA	3/5	0/5	1/5 5/5	2/5	5/5 2/5	3/5	2/5	5/5	1/5	1/5
AOSW	1/5	0/5	1/5 2/5	3/5	0/5 0/5	5/5	2/5	5/5	0/5	1/5
VFHO	0/5	5/5	0/5 3/5	0/5	2/5 1/5	3/5	0/5	4/5	0/5	4/5
PHO	2/5	4/5	1/5 5/5	2/5	2/5 0/5	3/5	4/5	5/5	2/5	4/5

*T DENOTES RABBITS INJECTED NEONATALLY.

**C DENOTES RABBITS INJECTED AT 8 WEEKS OF AGE.

† MEDIA ANTIGENS AND MYCOPLASMA STRAINS GROWN IN SUCH MEDIA.

‡ DENOTES NUMBER OF POSITIVE ANTISERA OVER NUMBER OF ANTISERA TESTED.

of the 25 antigens. However, in both cases the titers were relatively low.

A similar occurrence was demonstrated with two rabbits injected with AOSW broth medium (Table 7). One rabbit gave a positive titer to 10 of 25 antigens while the other rabbit's antiserum was only positive to 3 of the 25 antigens. Once again all titers were relatively low.

Table 7 shows that the control rabbits inoculated with media containing horse serum reacted to almost all the media antigens containing horse serum or which were grown in horse serum (39 positive of 45 tested). They did not react as strongly against the antigens associated with rabbit or swine sera with the exception of the rabbit inoculated with VFHO broth which gave a positive titer to all but one of the twenty five antigens. The rabbits injected with AOHO and PHO broth were positive only to 2 of 20 mycoplasma antigens grown on medium supplemented with rabbit or swine serum.

Serum samples taken at eight weeks of age before hyperimmunization injections began were tested by complement fixation against the four mycoplasma antigens. All C-F titers of these antisera were less than 32.

The final antiserum samples were then tested against all antigens by the gel diffusion precipitin test to aid in clarifying the cross reactions which occurred in the C-F test. The results of these tests are recorded in Tables 1-5. The number of positive antisera was greatly reduced; only 71 of 1300 tests were positive compared to 551 of 1300 tested by the C-F test. However, there appeared to be little correlation between the results of the two tests. The sera which were positive by gel precipitation had corresponding titers in the C-F test ranging from less than 32 through greater than 256; ten sera which were positive by gel

precipitation showed titers less than 32 in the C-F test. Thus, only 61 of the 551 positive C-F tests were confirmed by the gel precipitation tests. The extensive cross reactions which occurred in the C-F tests were not demonstrated by the gel precipitation tests.

A precipitation reaction occurred consistently between certain antisera which were in adjacent wells. Two typical examples are illustrated in Figure 1. Table 8 lists the antisera involved and the antigens with which lines of identity were obtained. The serum samples in the first column of Table 8 are those which showed some degree of tolerance to the media components by the C-F test while those in the second column showed no tolerance. Thus, all reactions appeared to involve tolerant versus non-tolerant antisera. Gel precipitation tests involving only tolerant antisera or only non-tolerant antisera were negative. Horse serum was the component involved in all of these reactions except for two pairs of antisera with AOSW as the common component. No lines of identity were established for the AOSW sera interactions.

TABLE 8--Antisera With Components Which Interacted in the Gel Diffusion Precipitin Test and Their Lines of Identity

TOLERANT ANTISERA	NON-TOLERANT ANTISERA	LINES OF IDENTITY ANTIGENS
VFHO	VFHO-C	VFHO, PHO, AOHO, B-600 (AOHO)
B-600 (VFHO) *	B-761 (VFHO)	VFHO, PHO, AOHO, B-762 (PHO)
VFHO	PG-31 (VFHO)	VFHO
B-761 (VFHO)	PG-31 (VFHO)	VFHO
B-600 (AOHO)	PG-31 (VFHO)	VFHO, PHO, AOHO, B-600(AOHO) B-762 (PHO)
PG-31 (AOHO)	PG-31C (AOHO)	AOHO, PHO, B-600 (AOHO)
AOHO	AOHO-C	AOHO, B-762 (PHO)
AOHO	PG-31C (AOHO)	AOHO, PHO, B-600 (AOHO)
AOSW	AOSW-C	
B-762 (AOSW)	AOSW-C	

*DENOTES ANTISERUM TO B-600 GROWN IN VFHO MEDIUM.

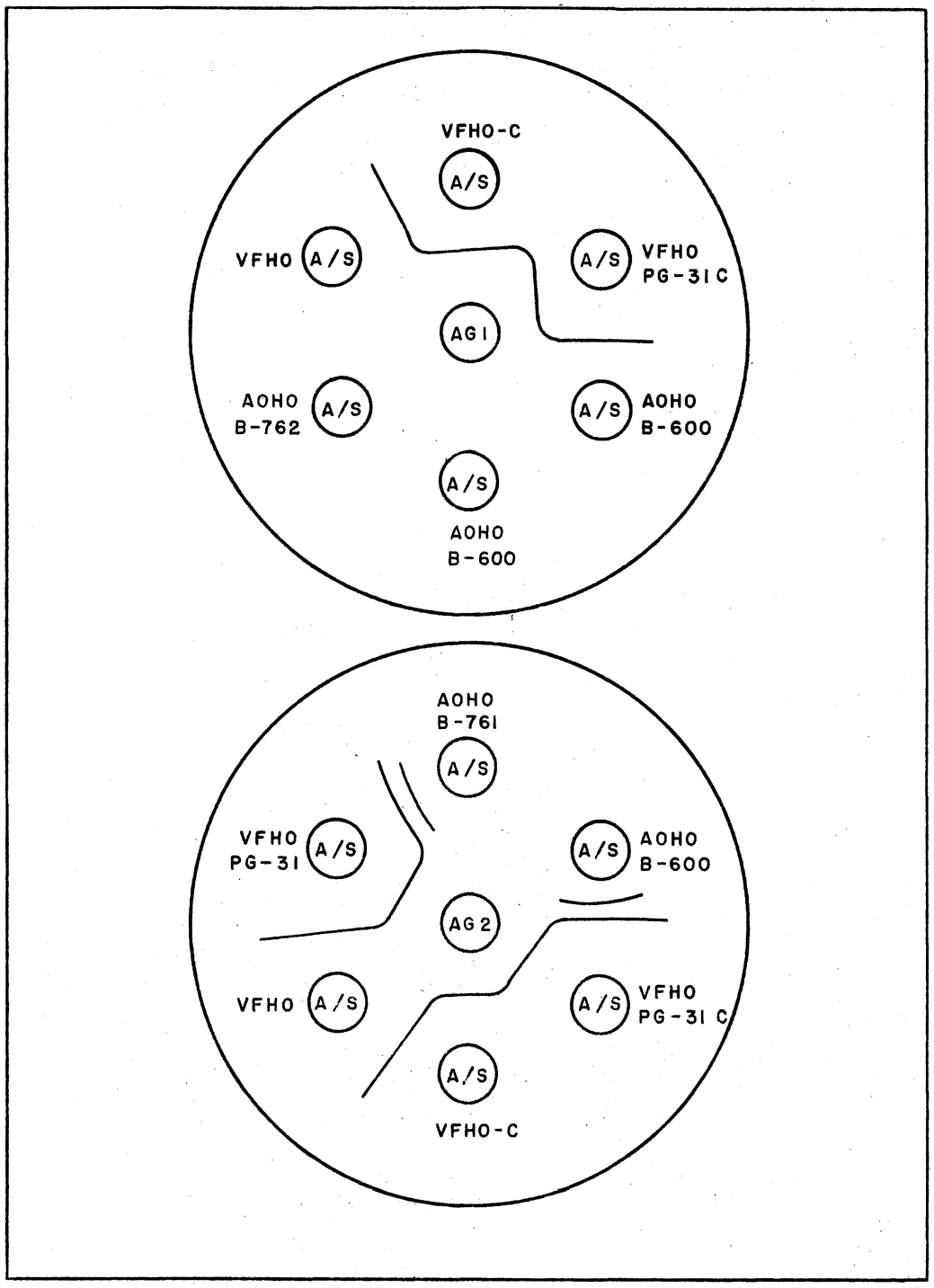

Fig 1--Gel diffusion precipitin test illustrating antisera interactions. Ag 1 = AOHO antigen; Ag 2 = VFHO antigen; A/S = antiserum.

DISCUSSION

The objective of this research was to produce tolerance in rabbits to media components so that serological cross reactions occurring in the complement fixation test would be the result of mycoplasma antigenic determinants rather than adsorbed media components. In those rabbits which appeared to develop tolerance to media components as recognized by the absence of C-F titers to the media antigens, cross reactions to hereologous strains were prevalent, and no consistent pattern of cross reactivity could be demonstrated.

The results of the C-F tests also demonstrated the inadequacy of the technique employed to produce tolerance. The injection of one 0.3 ml aliquot of broth medium containing 16% serum supplement within 24 hours after birth and one 0.5 ml aliquot of the same broth at 4 weeks of age was not sufficient to induce a state of unresponsiveness in a large enough percentage of rabbits to be routinely employed in the production of mycoplasma hyperimmune sera.

Golub and Weigle (10) have demonstrated that different doses of tolerogen are needed to produce an unresponsive state in different strains of inbred mice. They found that the variability between strains of mice was due to the efficiency with which the strains processed antigenic material, and the degree of efficiency appeared to be controlled by a genetic mechanism involving more than gene. The rabbits used in this experiment were of varied genetic background which may explain the variable immunological responses obtained from rabbits which had undergone

identical injections of the same material. Also, the rabbits were obtained from three different sources which meant that environmental influences, such as temperature and nutrition, which can affect antibody response, were not the same for all rabbits during their first eight weeks of life. The use of one inbred strain of rabbits raised under similar environmental conditions would be desirable in standardizing a method for producing tolerance.

Weigle (26) has produced evidence that multiple injections of small doses of protein antigens into neonatal rabbits are more effective than a single large dose. He was able to produce tolerance to a single antigen in 100% of the rabbits tested by injecting small aliquots of the antigen two times per week for a total of ten weeks. Since he used such a lengthy injection schedule, it would be desirable to determine the minimum amount of antigen and injections needed to produce tolerance to media components in 100% of a particular strain of rabbits to be used for hyperimmune sera production in order to determine whether it would be feasible to use such a method routinely.

An important consideration for production of tolerance in adult animals is the physical state of the antigen. Monomeric or deaggregated antigen, produced by high-speed centrifugation to remove aggregates, when injected into mature animals is known to cause a state of specific unresponsiveness, whereas the aggregated form will initiate an antibody response. The present theory explaining the phenomenon of tolerance (11) would suggest that in animals which are not immunologically competent, such as neonatal rabbits, it would not matter whether the antigen were in aggregated or deaggregated form. However, it may be worthwhile to compare the efficiency of both forms of antigen to produce tolerance in

neonatal rabbits.

One problem, involving the complement fixation test itself, was that some of the sera to be tested were anticomplementary. The reasons for serum becoming anticomplementary are not fully understood; contamination by bacteria is a known cause but not the sole cause. The antisera found to be anticomplementary in this experiment were not contaminated by bacteria. The amount of lipid present did not seem to alter the effect as the fasting of rabbits 18 to 24 hours before bleeding did not reduce the number of anticomplementary sera.

One important aspect associated with anticomplementary activity is the storage of serum. Serum can be stored at -20 °C. for at least three years without any significant change in antibody titer (6). However, at temperatures higher than -20 °C., changes may result leading to anti-complementary activity. The serum samples used in this experiment were stored in freezers at an average temperature of -10 °C. which may have led to the activity observed. However, there is still no explanation of why some sera become anticomplementary and some do not when they are handled in an identical manner.

The effect of repeated freezing and thawing may have had undesirable effects on the sera. The antisera used were thawed and refrozen many times; this could have been avoided by freezing small enough aliquots so that only the amount needed for one test at a time could be pulled out of storage. However, it becomes a difficult storage and packaging problem to divide antisera into tiny aliquots when handling such large amounts of sera as in this experiment. The most practical method would be to distribute the sera into as small aliquots as storage facilities would allow. This would decrease the number of freeze-thaw cycles.

Because it is not understood why serum becomes anticomplementary, the problem cannot be entirely avoided, and such activity is difficult to eliminate once it is present. There are many methods given in the literature for decreasing anticomplementary activity, although none are entirely effective, and most are laborious. It appears that this is a problem within itself, and its resolution would eliminate the discarding of such sera or their use only at high dilutions.

The large number of heterologous cross reactions seen in the C-F tests are difficult to explain, especially in situations where the sera had no detectable antibodies to medium components. One explanation could be the interaction of the medium and the mycoplasma causing conformational changes in the mcyoplasma organism resulting in new antigenic determinants being exposed which are common to all strains. Or the medium and mycoplasma could be complexing in such a way that new antigenic determinants are actually formed. These new determinants could be very similar among different strains of mycoplasma, the media being responsible for the common antigenicity, but altered in such a way that it would not react with antisera specific for that medium.

The cultures used in this experiment had been passaged a large number of times on artificial media. It would be interesting to compare the cellular proteins by polyacrylamide gel electrophoresis of a strain of mycoplasma freshly isolated with the same strain after subsequent passaging on artificial media. If differences could be established, and other strains were shown to undergo similar protein changes, a basis for the understanding of these non-specific cross reactions could be developed.

Low pH has been shown to increase the adsorption of serum proteins

to Mycoplasma gallisepticum, and if the organisms are kept in a medium with a pH greater than 5.5, no adsorption of media is detectable by the gel diffusion precipitin test (4). Similar studies should be done using the complement fixation test to see if medium adsorption, as detected by this test, could be avoided.

The gel diffusion precipitin test demonstrated the presence of media antigens in the sera of the tolerant rabbits. This is to be expected and confirms the tolerant state of the animal since it is known that the antigen does persist in the unresponsive animal (26). The presence of antigen in the sera should have had no effect on the results of the complement fixation test, nor should its presence alter the gel diffusion precipitin test except for the precipitation lines which form between adjacent tolerant and non-tolerant sera leading to extraneous and more complicated precipitation patterns.

SUMMARY

Rabbits injected within twenty four hours after birth with 0.3 ml of broth medium containing 16% serum supplement and at four weeks of age with 0.5 ml of the same broth medium varied in their ability to become tolerant to the media antigens. The number of rabbits which demonstrated tolerance by the complement fixation test was not sufficient to employ this method as a standard procedure in the production of mycoplasma hyperimmune sera.

The genetic and environmental disparity of the rabbits appeared to play the significant role in the variable responses attained, rather than the media differences.

Heterologous cross reactions were not eliminated by the use of sera from rabbits tolerant to media components. Further investigations regarding the alteration of mycoplasma antigenic determinants by prolonged cultivation on artificial media may explain some of these non-specific reactions.

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